2-Aminopyrimidine Derivatives as New Selective Fibroblast Growth Factor Receptor 4 (FGFR4) Inhibitors

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ABSTRACT: A series of 2-aminopyrimidine derivatives were designed and synthesized as highly selective FGFR4 inhibitors. One of the most promising compounds **2n** tightly bound FGFR4 with a K_d value of 3.3 nM and potently inhibited its enzymatic activity with an IC_{50} value of 2.6 nM, but completely spared FGFR1/2/3. The compound selectively suppressed proliferation of breast cancer cells harboring dysregulated FGFR4 signaling with an IC_{50} value of 0.38 μM. Furthermore, **2n** exhibited extraordinary target specificity in a Kinome-wide screen against 468 kinases, with S(35) and S(10) selectivity scores of 0.01 and 0.007 at 1.0 μM, respectively.

The fibroblast growth factor receptor (FGFR) family comprises four functional members (i.e., FGFR1, FGFR2, FGFR3, FGFR4), which consist of a conserved extracellular ligand-binding domain, a single transmembrane region and a cytosolic region with a split tyrosine kinase domain.[1-2] FGFRs are high affinity receptors for the fibroblast growth factors (FGFs) which includes 18 members.[1] FGF/FGFR signaling is tightly regulated by ligand specificity, and temporal and spatial expression of the signaling components, and is involved in many fundamental biological processes, such as metabolism, embryonic development and adult tissue homeostasis.[2-3]

FGFR4 is mainly expressed in liver, lung, lymphoid and breast tissues and specifically utilizes endocrine FGF19 as the intracellular ligand.[4-5] Upon binding with a complex formed by FGFR4 and its co-receptor, transmembrane β-Klotho protein,[4] FGFR19 promotes receptor dimerization, autophosphorylation and activation of the signaling pathway, which plays significant roles in regulation of bile acid homeostasis,[6] and maintenance of glucose and protein metabolism.[7] FGFR4 and/or FGF19 aberrations (i.e., amplification, gene fusion or mutation) have been recently detected in a variety of human cancers. For instance, FGF19 is amplified in approximate 15% of breast cancer,[8] and high expression of FGF19 was closely correlated to poor prognosis of the disease.[9] Various genetic alterations of FGFR4, such as amplification,[10] point mutation[11] and single nucleotide polymorphism,[12] were also detected in human breast tumor tissues. It has been demonstrated that silencing of FGF19/FGFR4 signaling by siRNA or introduction of an anti-FGF19 antibody potently suppresses proliferation and induces apoptosis of triple-negative breast cancer cells,[11, 13] offering promise in a disease setting that scarcely benefits from currently available therapies.[14] A synergistic interaction was also observed between FGFR4 signaling blockade and doxorubicin chemotherapy.[13, 15] Collective evidence also suggests that FGF19/FGFR4 signaling is an oncogenic driver in a subset of hepatocellular carcinoma (HCC) patients. In an analysis of 281 HCC patients, approximately 48% of the tumors expressed FGF19, which was associated with larger tumor size, more advanced stage and early recurrence.[16] In addition, about 30% of HCC samples tested display increased expression of FGFR4[17] and 25% overexpress β-Klotho protein.[18] These data collectively indicate that targeting the FGF19/FGFR4 signaling pathway may provide a new promising strategy for the management of a defined subgroup of cancer patients.[19,20]

A number of small molecule FGFR inhibitors with different selectivity profiles have been developed into clinical investigation for the management of FGFR-driven human cancers.[21] Unfortunately, hyperphosphatemia and tissue calcification are common "on-target" clinical adverse ef-

fects for most of the drugs investigated,[22-23] due to their strong inhibitory activity against FGFR1 and FGFR3, blocking FGF23-mediated signaling.[24] Given the fact that the FGFR family share significant sequence homology in their kinase domains (Figure 1A), it is a considerable challenge to develop selective FGFR4 inhibitors over the other family members. Most recently, BLU9931 (**1**, Figure 1B) was discovered as the first selective FGFR4 inhibitor. The structure-based design of this molecule involved the covalent targeting of a unique Cysteine 552 (Cys552) residue in the hinge region of the receptor (Figure 1C).[25] The compound exhibited extraordinary target specificity and demonstrated significant anti-proliferative activity against a panel of human hepatocellular carcinoma (HCC) cells with activated FGFR4 signaling. Moreover, the compound also demonstrated promising in vivo efficacy in several xenograft and patient-derived xenograft (PDX) models of HCC.[25] Most recently, BLU554, a structurally related derivative of inhibitor **1**, was advanced into early stage clinical investigation (clinical trial ID NCT02508467). The clinical report of the compound is eagerly awaited. Nevertheless, it is still highly valuable to identify new selective FGFR4 inhibitors for anticancer drug discovery. Herein, we describe the design, synthesis and biological evaluation of a series of 2-aminopyrimidine derivatives as new selective FGFR4 inhibitors.[26]

The X-Ray co-crystal structure of compound **1** with FGFR4 revealed that the compound binds with the ATP-binding pocket of the receptor, forming a critical covalent bond with Cys552 (Figure 1C). It was also shown that C_a, C_b, C_c and C_d atoms in this inhibitor were in an almost coplanar configuration (Figure 1B/C), and there were no obvious direct interactions between the phenyl ring A (Figure 1B) and the protein. It is well-known that the lowest energy

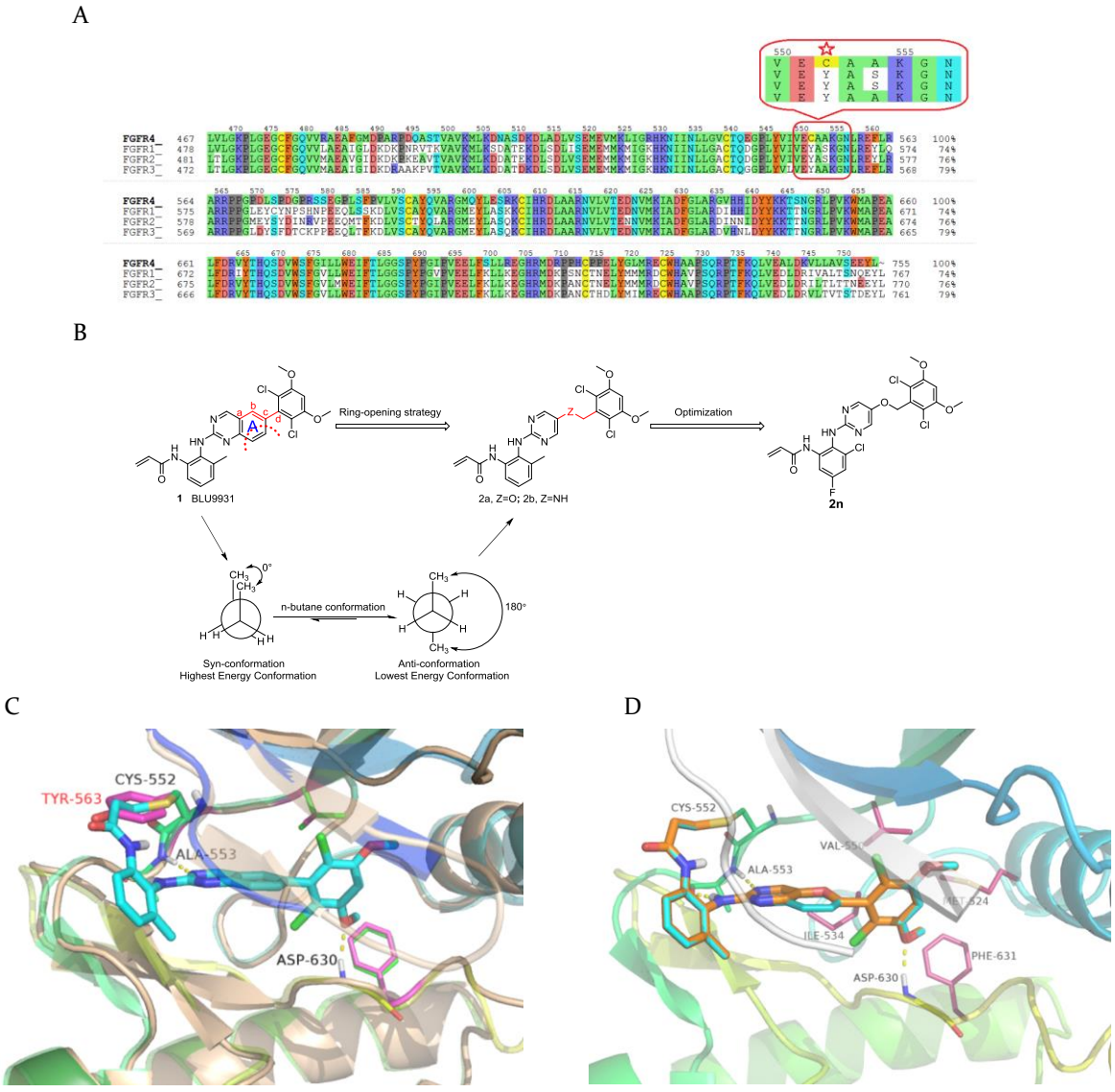
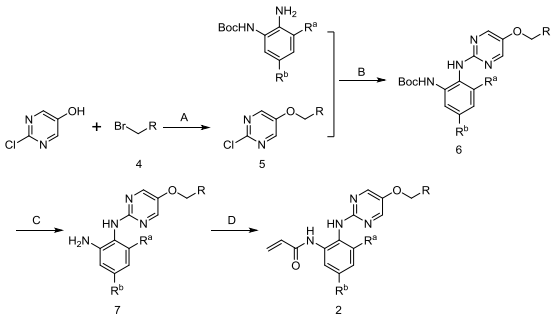


Figure 1. Design of new selective FGFR4 inhibitors. (A) Sequence alignment of the FGFR1/2/3/4 kinase domains using the multiple sequence viewer in Maestro. (B) Design of new selective FGFR4 inhibitors. (C) Superimposition of FGFR4-BLU9931 (PDB: 4xcu; multicolor; residues, green) with FGFR1 (PDB: 3tto; constant color, brown; residues, purple). (D) Predicted binding mode of 2a (orange, carbon atoms; blue, nitrogen atoms; red, oxygen atoms; green, chlorine atoms) in a crystal structure of FGFR4 (PDB:4xcu) with BLU9931 (cyan) aligned to it.

conformation of n-butane is an anti-form configuration where the dihedral angle of the four carbon atoms is approximately ±180° (Figure 1B).[27] Thus, an n-butane-like moiety might be utilized as a bioisostere of the phenyl ring A moiety to mimic the conformation of compound **1**. Based on this hypothesis, a series of 2-aminopyrimidine derivatives were designed and synthesized (Figure 1B). Encouragingly, a preliminary computational study suggested that compound **2a**, which harbors a methyleneoxyl moiety to potentially mimic the conformation of the parent compound **1**, nicely bound into the ATP-binding pocket of FGFR4 with a similar mode to that of compound **1** (Figure 1D). The warhead acrylamide is predicted to covalently react with the sulfhydryl side chain of Cys552, which derives the paralog selectivity.[25] The 2-aminopyrimidine core forms two pairs of hydrogen bonds with the backbone amino and carbonyl group of Ala553, respectively. The tetra-substituted phenyl group, which adopts an almost perpendicular orientation to the 2-aminopyrimidine core, makes favorable van der Waals contacts with the hydrophobic back pocket of the ATP binding site, with one methoxyl forming a hydrogen bond with the backbone carboxamide NH group of Asp630. It is also noteworthy that compound 2a possesses less aromatic ring count and higher Fsp^3 value than that of compound **1**, which might benefit its drug-like physico-chemical properties.[28-29]

Scheme 1. Synthesis of Compounds 2a-2z*



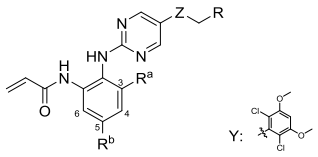
*Reagents and conditions: (A) K_2CO_3, $Bu_4N^+I^-$, DMF, 60℃, 2.0 h; (B) $Pd(OAc)_2$, XantPhos, Cs_2CO_3, 100℃, Overnight; (C) TFA, DCM, rt, Overnight; (D) Acryloyl chloride, DIEA, dry DCM, 0℃, 2.0 h. R: substituted phenyl.

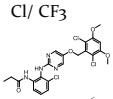
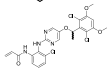
The designed inhibitors were readily prepared by using a Buchwald-Hartwig cross-coupling reaction as the key step (Scheme 1).[30] Briefly, commercially available 2-chloropyrimidin-5-ol was treated with different substituted benzyl bromides giving the corresponding intermediates **5** through a nucleophilic substitution reaction. Compounds **5** were coupled with various Boc-protected o-phenylenediamines under palladium catalysis to afford the Buchwald-Hartwig coupling products, which were deprotected to produce the arylamines **7**. Intermediates **7** were reacted with acryloyl chloride to produce the designed molecules **2a**, **2c-2g** and **2k-2l** with good to moderate yields. The other designed inhibitors were synthesized by utilizing a similar protocol (Supporting Information).

Kinase inhibitory activities of the designed compounds against FGFR4 and the family members FGFR1/2/3 were evaluated by using a well-established FRET-based Z'-Lyte assay.[25] The first reported selective FGFR4 inhibitor **1** and a FGFR1/2/3 inhibitor FIIN-1[31] were used as a positive control to validate the screening conditions. Under our assay conditions, it exhibited strong inhibitory potency against FGFR4 with an IC_{50} value of 6.0 nM, further to this compound **1** was significantly less potent against FGFR1/2/3, with our data comparing favorably to the reported data.[25] Whereas, the pan FGFRs inhibitor FIIN-1 exhibited IC_{50} values of 3.6, 2.3, 6.0 and 261.3 nM against FGFR1, FGFR2, FGFR3 and FGFR4, respectively. We were delighted to find that our first designed compound **2a** potently suppressed the enzymatic activity of FGFR4 with an IC_{50} value of 11.1 nM, which was only 2-fold less potent than the positive control **1**, yet entirely spared FGFR1/2/3 with IC_{50} values > 10,000 nM (Table 1). Investigation also suggested that the methyleneoxyl linker in compound **2a** could be replaced by a methyleneamino moiety (**2b**) to demonstrate slightly decreased FGFR4 suppression with an IC_{50} value of 43.4 nM, while maintaining the paralog selectivity. Compound **2a** harbored a methyl substitution at the 3'- position, which was adopted from the original parental molecule **1** and was considered to be important for the selective inhibition against FGFR4. Thus, we first investigated the potential impact of the methyl substituent position on the paralog sparing FGFR4 inhibition. It was shown that this substitutent position indeed had a great influence on the target inhibitory potency and paralog selectivity. When the methyl group was shifted to the 6'- position, the resulting molecule **2c** demonstrated a total loss of all FGFR inhibitory activity. The 5'-methyl substituted compound **2d** exhibited slightly increased FGFR4 inhibitory potency with an IC_{50} value of 7.5 nM, but its selectivity was decreased due to increased inhibition of FGFR1. Whereas, a 4'- methyl substitutent (**2e**) resulted in a slight decrease in FGFR4 inhibitory potency and similar FGFR1 inhibition as that observed in the 5'-position (**2d**). Although removal of the methyl group barely affected the FGFR4 inhibition potency, the resulting molecule **2f** displayed obviously improved suppression against both FGFR1 and FGFR2 with IC_{50} values of 431.0 and 311.9 nM, respectively, suggesting significant loss of its target specificity. Thus, the results collectively suggested that 3'- substitution is an optimal strategy for designing new selective FGFR4 inhibitors. Further investigation also revealed that the 3'- position is well tolerated to a variety of substituted groups such as ethyl (**2g**), methoxyl (**2h**), chloro- (**2l**) and trifluoromethyl (**2m**) moieties which demonstrated potent and paralog-selective inhibition of FGFR4. Encouragingly, the 3'-chloro substituted analogue **2l** exhibited an obviously improved FGFR4 inhibition potency with an IC_{50} value of 3.8 nM, but retained excellent selectivity against FGFR1/2/3 at a high concentration of 10,000 nM. Not surprisingly, the 3'-fluro substituted compound **2k** displayed a similar potency and selectivity profile to that of the 3'-unsubstituted molecule **2f**. Notably, increases in size of a 3'-alkoxy group resulted in stepwise loss of FGFR4 inhibitory potency (compare **2h**, **2i** and **2j**). Our investigation suggested that substitution at the 5'- position of the molecules could retain strong FGFR4 inhibition. Thus, several 3', 5'- disubstituted compounds,

i.e., **2n**, **2o**, **2p**, were also designed and synthesized. These displayed potent and selective suppression of FGFR4. Particularly, the 3'-chloro-5'-fluoro- analogue **2n** which exhibited the best FGFR4 inhibitory activity with an IC_{50} value of 2.6 nM, 2-fold more potent than compound **1**. Compound **2n** also displayed an obviously improved paralog selectivity over the reported inhibitor **1**. Further investigation also revealed that warhead acrylamide was crucial for potent FGFR4 inhibition. Replacement of the acrylamide with an α, β-saturated propionamide moiety (**2q**) caused a totally abolishment of FGFR inhibition. It is generally accepted that a benzyloxy group possesses a potential metabolic liability and introduction of steric hindrance is a feasible strategy to minimize oxidative metabolism and improve the metabolic stability of the molecule. Based on this rationale, compound **2r** in which a methyl group was introduced at the benzyloxyl linker, was designed and synthesized. Disappointingly, this modification caused a dramatic 220-fold loss in potency. Modification was also conducted on the tetra-substituted phenyl moiety which was predicted to approach the hydrophobic back pocket of the ATP binding site. It was shown that the tetra-substitution was critical for the molecules to demonstrate strong FGFR4 inhibition. Removal of one or more substituents caused significant potency loss against FGFR4. For instance, removal of a methoxyl group (**2s**) resulted in a 6.3-fold potency loss, whereas deletion of a chloro- group (**2t**) reduces potency 3-fold. The other chloro- or methoxyl deleted compounds (**2u-2z**) displayed a 6.0 to 122-fold loss of potency. Thus, compounds **2a**, **2h**, **2l**, **2o**, and **2n** stood out as representatives for further investigation due to their strong FGFR4 inhibitory potency and extraordinary paralog specificity.

Table 1. Kinase inhibitory activities of compounds **2a-2z**



Compd	Z	R^a/R^b	R	Kinase inhibitory activity* (IC_{50}, nM)			
				FGFR1	FGFR2	FGFR3	FGFR4
2a	O	Me/H	Y	>10,000	>10,000	>10,000	11.1
2b	N	Me/H	Y	>10,000	>10,000	>10,000	43.4
2c	O	6'-Me	Y	>10,000	>10,000	>10,000	>10,000
2d	O	5'-Me	Y	825.3	>10,000	>10,000	7.5
2e	O	4'-Me	Y	975.1	>10,000	>10,000	28.1
2f	O	H/H	Y	431.0	311.9	1,200	8.2
2g	O	Et/H	Y	>10,000	>10,000	>10,000	25.6
2h	O	MeO/H	Y	>10,000	>10,000	>10,000	4.8
2i	O	EtO/H	Y	>10,000	>10,000	>10,000	72.9
2j	O	iPrO/H	Y	>10,000	>10,000	>10,000	202.3
2k	O	F/H	Y	617.7	396.8	4,300	5.3
2l	O	Cl/H	Y	>10,000	>10,000	>10,000	3.8
2m	O	CF_3/H	Y	>10,000	>10,000	>10,000	24.9
2n	O	Cl/F	Y	>10,000	>10,000	>10,000	2.6
2o	O	Cl/ Cl	Y	>10,000	>10,000	>10,000	8.5
2p	O	Cl/ CF_3	Y	>10,000	>10,000	>10,000	17.1
2q				>10,000	>10,000	>10,000	>10,000
2r				>10,000	>10,000	>10,000	669.2
2s	O	Cl/H		>10,000	>10,000	>10,000	24.0
2t	O	Cl/H		>10,000	>10,000	>10,000	12.3
2u	O	Cl/H		>10,000	>10,000	>10,000	38.7
2v	O	Cl/H		>10,000	>10,000	>10,000	152.4
2w	O	Cl/H		>10,000	>10,000	>10,000	98.0
2x	O	Cl/H		>10,000	>10,000	>10,000	22.7
2y	O	Cl/H		>10,000	>10,000	>10,000	273.5
2z	O	Cl/H		>10,000	>10,000	>10,000	465.7
1				239.9	246.9	243.5	6.0
FIIN-1				3.6	2.3	6.0	261.3

*Kinase activity assays were performed using a FRET-based Z'-Lyte biochemical assay.[25] Data are means of three independent experiments, and the variations are <20%.

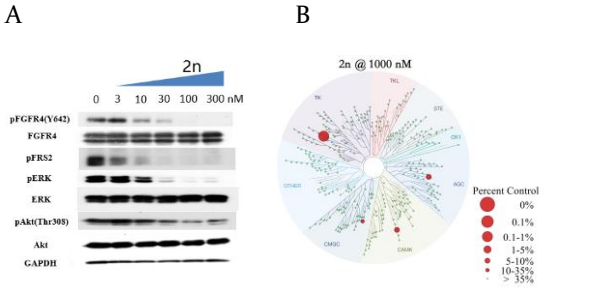


Figure 2. Compound **2n** selectively targets FGFR4. (A) Compound 2n dose-dependently inhibits the activation of FGFR4 and downstream proteins in MDA-MB-453 cells with $FGFR4^{Y367C}$. The cells were treated with or without compound 2n for 4h at the indicated concentrations, respectively. Cells were then harvested for Western blot analysis. (B) KinomeScan kinase selectivity profile for compound **2n**. Compound **2n** was profiled at a concentration of 1.0 μM against a diverse panel of 468 kinases by DiscoveRx.

Taking compound **2n** as an example, the strong FGFR4 inhibition of the compounds was further validated by investigating its inhibition of the activation of FGFR4 and downstream signaling partners in MDA-MB-453 cells harboring an activating $FGFR4^{Y367C}$ mutation (Figure 2A).[11] It was shown that the compound diminished the phosphorylation of FGFR4 and inhibited the activation of downstream FRS2, ERK1/2 and AKT in a dose-dependent manner, while the total amount of corresponding proteins remained unchanged as determined by western blot analysis. The phosphorylation of FGFR4, ERK1/2 and FRS2 was almost totally abolished after a treatment of 0.03 μM of compound **2n**, although its effect on p-Akt was less obvious.

The binding affinity of compound 2n for FGFR4 was also determined by using an active-site-dependent competition binding assay (DiscoveRx Corporation, San Diego, USA). It

was shown that compound **2n** tightly bound to the ATP-binding site of the kinase with a binding constant (K_d) value of 3.3 nM, validating its strong kinase inhibition against FGFR4. We further profiled the target specificity of this compound against a panel of 468 kinases (including 403 nonmutated kinases) using the DiscoveRx screening platform at a concentration of 1.0 μM, which was about 300 times higher than its K_d value with FGFR4. The results revealed that **2n** demonstrated impressive target specificity with S(10) and S(35) selectivity scores of 0.007 and 0.01 at 1.0 μM, respectively (Table S2). For instance, **2n** showed almost 100% competition rate (99.5% inhibition, ctrl% = 0.5) with FGFR4 at 1.0 μM, and only showed obvious binding to a very small portion of the kinases investigated. The main "off-target" hits included calcium/calmodulin dependent protein kinase ID (CAMK1D, 94.7% inhibition), G-protein coupled receptor kinase 4 (GRK4, 92.8% inhibition), and SRSF Protein Kinase 3 (SPRK3, 88% inhibition).

Table 2. Antiproliferative activities of the designed compounds against a panel of breast cancer cells

Compd	Cell growth inhibition (IC_{50}, μM)		
	MDA-MB-453[a]	MDA-MB-231[b]	MCF-7[b]
2a	0.22 ±0.13	>10	>10
2h	0.21 ±0.16	2.14 ±1.08	1.18 ±0.89
2l	0.65 ±0.42	>10	>10
2o	0.87 ±0.75	>10	>10
2n	0.38 ±0.17	>10	>10
1	0.32 ±0.14	3.76 ±2.35	2.34 ±0.88

[a] Cancer cells harboring an $FGFR4^{Y367C}$ mutation that also demonstrate overexpression of FGFR4. [b]Cancer cells that have low FGFR4 expression under IP-Western blotting. The IC_{50} values were determined using CCK8. Data are mean values ± standard deviation (SD) of three independent experiments.

The growth inhibitory effects of the compounds **2a**, **2h**, **2l**, **2o**, and **2n** were evaluated against a panel of breast cancer cell lines (Table 2). MDA-MB-453 breast cancer cells predominantly express a mutated form of FGFR4, $FGFR4^{Y367C}$, which causes spontaneous receptor dimerization and constitutive activation in a ligand-independent manner.[11] Moreover, $FGFR4^{Y367C}$ had been identified as an important driver gene for the proliferation of MDA-MB-453 cells. On the another hand, MCF-7 and MDA-MB-231 breast cancer cells harbor low level of FGFR4 and serve as negative control cell models to investigate the target specificity of the inhibitors.[13] It was shown that almost all of the selected FGFR4 inhibitors exhibited promising antiproliferative activity against MDA-MB-453 breast cancer cells with similar potencies to that of inhibitor **1**. However, none of them displayed obvious cell growth inhibition against MCF-7 and MDA-MB-231 breast cancer cells expressing low levels of FGFR4. For instance, compound **2n** potently inhibited the proliferation of MDA-MB-453 breast cancer cells with an IC_{50} value of 0.38 μM, which is comparable to that of compound **1**, but was unable to suppress the growth of MCF-7 and MDA-MB-231 cancer cells at a 10 μM concentration. These data further supported the strong and selective antagonism of the new inhibitors against FGFR4. It is also noteworthy that all of the new inhibitors appear to display better target specificity than the original compound **1** in the cell based assays, which is consistent with the data from the biochemical kinase inhibition assays. Further investigation also demonstrated that compound **2n** dose-dependently induced apoptosis in MDA-MB-453 cells (Figure S2).

In summary, a series of 2-aminopyrimidine derivatives were designed and synthesized as highly selective FGFR4 inhibitors. These compounds potently suppressed FGFR4 activity with low nanomolar IC_{50} values and excellent isotype selectivity. One of the most promising compounds **2n** tightly bound with FGFR4 with a K_d value of 3.3 nM and potently inhibited its enzymatic activity with an IC_{50} value of 2.6 nM, but completely spared FGFR1/2/3. The compound selectively suppressed proliferation of breast cancer cells harboring dysregulated FGFR4 signaling with an IC_{50} value of 0.38 μM. Furthermore, it exhibited extraordinary target specificity in a Kinome-wide screen against 468 kinases, with S(10) and S(35) selectivity scores of 0.007 and 0.01 at 1.0 μM, respectively. Compound **2n** may serve as new lead compound for future anticancer drug discovery. Further structural optimization of the compound and *in vivo* antitumor efficacy investigation are ongoing.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the synthesis, 1H NMR and 13C NMR for final compounds, kinase selectivity, and details of in vitro assays.

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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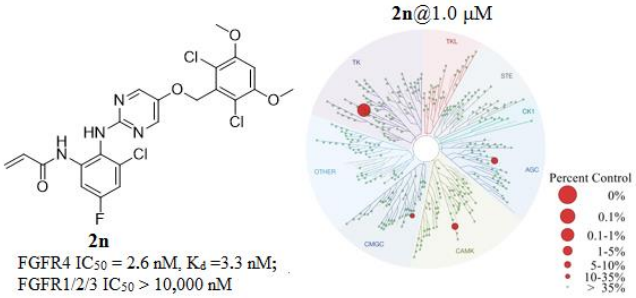

2n

FGFR4 IC_{50} = 2.6 nM, K_d =3.3 nM;
FGFR1/2/3 IC_{50} > 10,000 nM